



TITLE:

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CITATION:

Tanaka, Shinji ...[et al]. Chaperonin-encapsulation of proteins for NMR.. Biochimica et biophysica acta 2010, 1804(4): 866-871

ISSUE DATE:

2010-04

URL:

<http://hdl.handle.net/2433/108262>

RIGHT:

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## Chaperonin-Encapsulation of Proteins for NMR

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**Keywords:** aggregation, NMR, molecular chaperone, GroE, protein encapsulation

## Abstract

A novel chaperonin-encapsulation system for NMR measurements has been designed. The single-ring variant SR398 with an ATPase deficient mutation of GroEL, also known as chaperonin, bound co-chaperonin GroES irreversibly, forming a stable cage to encapsulate a target protein. A small GroEL-binding tag made it possible to perform all steps of the encapsulation under near physiological conditions while retaining the native conformation of the target protein. About half of the SR398/GroES cages encapsulated target protein molecules. As binding only depends on the 12-residue tag sequence, this encapsulation method is applicable to a large number of proteins. Isolation of the target proteins in the molecular cage of chaperonin will allow the study of highly aggregation-prone proteins by solution NMR.

*Abbreviations:* NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum coherence; SR398, single-ring mutant of GroEL with ATPase deficient mutation D398A; SBP, strongly binding peptide to GroEL

## 1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy is one of the most powerful techniques for the analysis of the structure, dynamics and function of proteins in solution. It is also widely used to study protein-protein and protein-ligand interactions. Recent advances in isotope labeling, TROSY-based pulse sequences and sensitivity have made large proteins amenable to NMR studies even at low concentrations [1–3]. A frequent problem, however, is presented by the formation of nonspecific aggregates that lead to excessively broad NMR signals due to their high molecular weight or to chemical exchange between different states of aggregation. Many of these ill-behaved proteins are not amenable to NMR studies in solution.

To overcome intermolecular self-association, the protein molecules must be kept apart. A recent report by Lazar *et al.* showed that aggregate formation of fibrillogenic peptides can be prevented by encapsulating them in single bilayer vesicles [4]. Wand and co-workers used reverse micelles to sequester target proteins in organic solvents of low viscosity [5,6]. Although these systems can, in principle, keep self-associating protein molecules apart, the size of the aqueous cavities is quite adaptable, making it difficult to produce homogenous preparations of singly encapsulated molecules. Furthermore, proteins easily unfold during sample preparation in these lipid-based systems, which may be problematic for proteins that do not refold reversibly, and it is difficult to analyze the effects of added ligand compounds, because lipids are impermeable to most water-soluble compounds. A better system would be a semi-permeable shell or cage of defined size, which allows the translocation of small compounds across the wall while preventing unwanted protein-protein interaction.

Here we show that the *E. coli* GroEL/GroES chaperone system, also known as

chaperonin, presents a suitable cage for trapping protein molecules as monomers or low-molecular weight oligomers where they are amenable to analysis by NMR spectroscopy. The combination of ATPase-deficient mutant of GroEL and small peptide sequence with high affinity for GroEL, made it possible to encapsulate a model substrate protein, ubiquitin, within the cavity of GroEL/GroES cage. Importantly, all the procedures were performed under near physiological conditions, indicating it is possible to encapsulate any target protein using the same method while avoiding denaturation of the target protein that is often irreversible. Our method would also be useful to examine the interaction of the target protein with its ligand because of the semipermeable character in GroEL/GroES cage.

## 2. Materials and Methods

### 2.1. Protein expression and purification

The expression vector for SR398 was constructed by introducing the D398A mutation in the expression plasmid for the single-ring mutant of GroEL (pEL-SR1, containing mutations R452G/E461A/S463A/V464A), which was obtained as a gift from Dr. K. Kuwajima [7,8]. The resulting plasmid, pEL-SR398, was transformed into the *E. coli* strain BL21(DE3)/pLysS (Novagen). Expression and purification of SR398 and co-chaperone GroES were carried out as described before with slight modification [9,10]. The entire purification was carried out at 4°C in 50 mM Tris-HCl (pH 7.8) containing 10% (w/v) glycerol, 5 mM mercaptoethanol, 25 mM NaCl and 1 mM EDTA (buffer TGMN). After lysis and removal of insoluble materials by centrifugation, ammonium sulfate was added to a concentration of 55% saturation. The precipitated protein was collected loaded onto Sephacryl S-300 ( $\phi$  2.6 × 100 cm; GE Healthcare).

The fractions of proteins were pooled, and loaded separately onto a Q-Sepharose anion-exchange column ( $\phi$  2.6  $\times$  20 cm; GE Healthcare). The proteins were eluted with a linear gradient of 0–1 M NaCl in buffer TGMN. Purity of the protein was examined to be >90% by SDS-PAGE stained with Coomassie Brilliant Blue R-250.

The expression plasmid for the substrate protein, ubq-SBP, was constructed from a pET vector encoding the human ubiquitin gene with a C-terminal His6-tag (pET-ubq, encoding the sequence  $^1\text{M}\dots\text{G}^{76}\text{-SHHHHHH}^{83}$ ). SBP was added by C-terminal extension using PCR. The resulting plasmid, pET-ubqSBP, encoded ubiquitin with the sequence  $^1\text{M}\dots\text{G}^{76}\text{-SHHHHHH-CGGG-SWMTTPWGFLHP}^{99}$ . The pET-ubq and pET-ubq-SBP plasmids were transformed into BL21(DE3)/pLysS and expressed in LB or M9 media with  $^{15}\text{NH}_4\text{Cl}$  containing 50  $\mu\text{g/mL}$  of ampicillin. The proteins were purified by affinity chromatography with HisTrap FF (GE Healthcare) using a linear gradient of 20–500 mM of imidazole in 300 mM NaCl and 50 mM Na-phosphate (pH 7.5).

## 2.2. Ternary complex formation

Encapsulation of ubq-SBP into the molecular cage of SR398/GroES was performed as follows. First, SR398 and ubq-SBP were dialyzed against buffer A (50 mM Na-phosphate (pH 7.0), 200 mM NaCl, 50 mM KCl, 10 mM  $\text{MgCl}_2$ , 5 mM  $\beta$ -mercaptoethanol), then mixed and incubated for 5 min. GroES in buffer A and ATP were added to the mixture of SR398 and ubq-SBP, and incubated for 5 min. Final concentrations in the mixture were 15  $\mu\text{M}$  SR398, 30  $\mu\text{M}$  GroES, 150  $\mu\text{M}$  ubq-SBP, and 2 mM ATP. The mixture was loaded onto Sephacryl-S300 (GE Healthcare, 9  $\times$  125 mm) equilibrated with buffer A containing 2 mM ATP, and 200  $\mu\text{L}$  fractions were collected.

Each fraction was analyzed by tricine-SDS-PAGE [11] and proteins were detected by silver staining (Wako Pure Chemical Industries). The fractions containing ternary complex (fractions 14–17) were concentrated by ultrafiltration (Millipore, 10 kDa molecular weight cutoff (MWCO)) and analyzed quantitatively by reversed-phase HPLC using a column of Protein-R (Nacalai Tesque, 4.6 x 150 mm).

### 2.3. NMR measurements

The ternary complex with  $^{15}\text{N}$ -labeled ubq-SBP was produced essentially as described above, using buffer A containing 2 mM ATP and 6%  $\text{D}_2\text{O}$ . The protein mixture (19.8  $\mu\text{M}$  SR398, 39.6  $\mu\text{M}$  GroES, 198  $\mu\text{M}$   $^{15}\text{N}$ -ubq-SBP) was treated with Ni-Sepharose 6 Fast Flow (GE Healthcare) to remove unbound  $^{15}\text{N}$ -ubq-SBP. The concentration of any remaining free  $^{15}\text{N}$ -ubq-SBP was decreased further by washing 10 times with buffer A containing ATP and  $\text{D}_2\text{O}$ , using a filter membrane of 100 kDa MWCO. The spectrum was recorded at 37°C on a Bruker 600 MHz NMR spectrometer. A series of 16  $^{15}\text{N}$ -HSQC spectra (each with a measurement time of 160 minutes) was recorded. After confirming the identity of each spectrum, all spectra were combined into a single spectrum that corresponded to a total measurement time of 42.7 h. After the NMR measurement, the protein concentration in the sample was determined by reversed-phase HPLC to be 17  $\mu\text{M}$  of  $^{15}\text{N}$ -ubq-SBP and 78  $\mu\text{M}$  of SR398, corresponding to a molar ratio of 0.2:1. The reference spectrum of  $^{15}\text{N}$ -ubq-SBP in solution was generated by combining four sets of HSQC spectra with identical acquisition parameters as for ternary complex. The protein concentration in the reference sample was determined to be 62  $\mu\text{M}$ . The 4 spectra were summed to obtain a single reference spectrum. The spectra were analyzed using NMRPipe [12].

### 3. Results and discussion

#### 3.1. Chaperonin cycle and design of model substrate protein

GroEL is a large, homo-oligomeric protein composed of two heptameric rings of 57 kDa subunits stacked back to back. Each GroEL ring possesses a large central cavity with a hydrophobic entrance for the binding of substrate proteins in nonnative conformations [13,14]. Co-chaperonin GroES, a dome-shaped heptameric protein composed of 10 kDa subunits, binds to the GroEL ring in the presence of ATP. Binding of GroES leads to a large conformational change in GroEL, resulting in the encapsulation and release of the nonnative substrate protein into the GroEL-ES cavity. This so-called *cis* ternary complex acts as a folding chamber where substrate proteins can refold without interference from intermolecular aggregation [7,15–18]. Hydrolysis of ATP in the *cis* ring and ATP binding in the opposite (*trans*) ring of GroEL cause dissociation of GroES and release of substrate protein into solution [15–18]. We speculated that the *cis* ternary complex could be sufficiently stable to encapsulate proteins in their native conformation for the duration of NMR experiments (Figure 1).

To obtain a stable *cis* ternary complex, we used a single-ring mutant of GroEL with the ATPase-deficient mutation D398A. The mutant, referred to as SR398, is known to bind GroES stably because its dissociation is normally triggered by binding of ATP to the *trans*-ring (which is absent from SR398) and ATP hydrolysis in the *cis*-ring [17]. In addition, the single-ring GroEL has been shown to be capable of binding a substrate protein in non-native form and, with the help of GroES, capture a substrate within its large central cavity. Next, to make a stable complex with substrate protein, we used the 12 residue peptide fragment “strongly binding peptide” (SBP), that binds to the



substrate recognition site of GroEL with high affinity ( $K_D \sim 2 \mu\text{M}$ ) [19,20]. To demonstrate the generality of our method, we chose ubiquitin as a model substrate protein. Ubiquitin is a highly stable protein that refolds reversibly, and it has never been reported to interact with GroEL.

### 3.2. Ternary complex formation confirmed by SDS-PAGE

First, we confirmed the interaction of ubiquitin tagged with SBP sequence (ubq-SBP) with SR398 and GroES. The molecular weights of ubq-SBP and SR398 are 11.4 and 400 kDa (as a heptamer), respectively. When each of these proteins was subjected separately to a Sepharacryl S-300 size-exclusion column, they eluted as separated peaks with retention volume of 7 mL and 3 mL, respectively (data not shown). On the other hand, when the mixture of SR398, GroES and ubq-SBP was loaded to the column equilibrated with the buffer containing 2 mM ATP, a significant band corresponding to ubq-SBP was detected by tricine-SDS-PAGE in fractions where SR398 was eluted (fractions 14–17 in Figure 2A). In contrast, ubiquitin free from SBP-tag sequence did not measurably interact with SR398, indicating the interaction was specifically and depended on SBP tag sequence (Figure 2C). This is rather consistent with general observations that GroEL specifically recognizes and binds to substrate proteins in denatured states. These results demonstrated the formation of a stable ternary complex of SR398, GroES and ubq-SBP. It should be noted that complex formation was achieved under near physiological conditions and there was no evidence of denaturation of ubq-SBP throughout the experiments.

In principle, each subunit of GroEL has a binding site for the SBP tag so that up to 7 molecules of SBPs could bind to SR398. Indeed, it has been reported that all 7

binding sites were occupied by SBPs in X-ray crystallographic study [19,20]. To determine how many molecules of ubq-SBP were encapsulated in a SR398/GroES cavity, we collected fractions of size-exclusion chromatography corresponding to ternary complex and analyzed quantitatively by reversed-phase HPLC. By comparing the eluted peak area with separately applied protein solutions with known concentration, it was revealed that about 0.4 molecule of ubq-SBP per molecule of SR398 could be detected in isolated ternary complex fractions (Fig. 2B). This result appears to be largely due to steric hindrance between ubq-SBP and GroES, probably due to competition between them because both of them are expected to bind to the same apical domain of GroEL [19,20]. Indeed, much higher stoichiometric ratios were observed during formation of binary complexes between ubq-SBP and SR398 alone (data not shown). While sub-stoichiometric ratios probably leave some of the SR398/GroES cages unoccupied, they also promote the capture of single monomeric molecules. No peak corresponding to the substrate protein could be detected in the case of ubiquitin without SBP sequence, confirming again SBP sequence is necessary for the binding to GroEL (Fig. 2D).

### 3.3. NMR study of ubq-SBP encapsulated within chaperonin cage

Based on the above observation of stable ternary complex formation by ubq-SBP, SR398 and GroES, we prepared the ternary complex using  $^{15}\text{N}$ -labeled ubq-SBP for NMR measurement. By encapsulating an isotopically labeled substrate protein within the cage formed by unlabeled chaperonin, it is possible to monitor specifically the conformation of ubq-SBP without interference of signals from a huge chaperonin cage.

The  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectrum of the complex showed well-resolved cross-peaks

characteristic of protein in the native tertiary fold. Comparison with the corresponding spectrum recorded of the free protein in solution revealed close coincidence of most cross-peaks (Figure 3). This demonstrates that the SR398/GroES cage encapsulates the model substrate protein ubq-SBP in its native conformation. Close comparison of these spectra, however, revealed that several cross-peaks including those for SBP sequence could not be observed. This might be due to a possible interaction of hydrophobic SBP region with the apical domain and/or inner wall of SR398/GroES cage.

It is known that transverse relaxation time ( $R_2$ ), and line width of the signal in NMR spectrum depends on the fluctuations of environment surrounding each nuclear spin. Therefore, it reflects sensitively the local and global motion of the molecule. To assess the effect of encapsulation into a limited volume of space on the molecular motion, we analyzed line widths of several well resolved peaks. Figure 4 shows the horizontal slices taken from spectra in Fig. 3. By fitting each resonance line to a Lorentzian line shape, it was revealed that most of the amide resonances of ubiquitin in the cage were about twice as broad as those of free ubiquitin in solution. This indicates that the tether to the SR398/GroES cage slows the reorientational motions of ubiquitin only about two-fold.

### 3.4. Implications for chaperonin function

Functional role of chaperonin has been considered to serve as the “Anfinsen cage”, that is, it sequesters a substrate protein inside the cavity where the substrate refolds to the native structure determined by its amino acid sequence. However, there are many arguments whether chaperonin just isolates a substrate within its cavity, or it actively interacts with the substrate to facilitate the folding. The present study encapsulated a

protein in the native conformation by tagging with SBP, and it might not necessarily reflect the true interaction between chaperonin and its substrate, however, it would be noteworthy to consider the environment inside the cavity because the present study provided the first high quality NMR spectrum of the protein encapsulated within the central cavity of GroEL/ES.

Conformation of true substrate protein captured by GroEL, has already been analyzed by 2D NMR, in which the “binary complex” between human dihydrofolate reductase and single ring variant SR1 was formed [21]. The [ $^{15}\text{N}$ ,  $^1\text{H}$ ]-CRINEPT-HMQC spectrum of  $^{15}\text{N}$ -DHFR tightly bound to SR1 showed a very small chemical shift dispersion, indicating no stable secondary and tertiary structures, and large line widths due to slow overall tumbling of huge complex. These results were strikingly contrast to the cis-ternary complex of ubq-SBP, SR398 and GroES presented here, emphasizing the critical role of the cis-ternary complex in function of chaperonin.

Weissman et al. [15] reported that acid-denatured green fluorescent protein (GFP) recovered its characteristic fluorescence in the complex with single-ring variant of GroEL (SR1) and GroES. They analyzed the spectra and lifetime of fluorescence and concluded that GFP attained a native conformation within the cavity of chaperonin. Whereas their conclusion is, in a strict sense, limited around the region of fluorophore of GFP, we demonstrated here that the conformation of ubiquitin within the cavity of chaperonin was the same throughout the molecule as that in solution. On the other hand, from the analysis of fluorescence anisotropy, they concluded that the rotational correlation time of GFP molecule increased by about 4 times. Similar but less significant effect was found in the case of ubiquitin encapsulated in chaperonin. We obtained the results of about two times increase in the line width of  $^1\text{H}$  resonances,

which corresponds roughly twice increase in the rotational correlation time. Considering the relative dimensions of ubiquitin molecule ( $19,000 \text{ \AA}^3$ ) to that of chaperonin cage ( $175,000 \text{ \AA}^3$ ), these increases in the rotational correlation time might be consistent with each other.

Although line width of NMR signal is influenced by many factors including local fluctuations in environment surrounding the nuclear spin, we considered it might be attributable to the increase in the overall rotational correlation time of the molecule because of relatively homogeneous in most of resonances (Fig. 4). We cannot exclude the possibility of nonspecific interaction of hydrophobic SBP moiety with the apical domain of GroEL and/or the inside wall of the cis-cavity. However, these interactions are considered to be relatively small, because substrate tightly bound to the apical domain would have much broader line widths [21]. In addition, a repulsive interaction has been suggested for a majority of the substrate proteins and the interior wall of the cavity [22].

In summary, we successfully encapsulated a protein in the central cavity of the SR398/GroES complex. A small GroEL-binding tag is sufficient for specific recognition of a protein by SR398 and can lead to successful encapsulation of about 50% of the target protein molecules in the folding chamber of SR398/GroES. Importantly, all steps of the encapsulation were performed under near physiological conditions, retaining the native conformation of the target protein. As binding only depends on the 12-residue tag sequence, this encapsulation method can be applied to a large number of target proteins. Isolation of the target proteins in the molecular cage of SR398/GroES will make it possible to study highly aggregation-prone proteins by solution NMR.

## Acknowledgements

This work was supported by Grants-in-Aid for Young Scientists (A) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and by the Takeda Science Foundation.

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## Figure legends

**Figure 1.** Schematic drawing of (A) the ATP-dependent chaperonin-cycle of GroEL and GroES and (B, C) the chaperonin-encapsulation system for NMR measurements designed in the present work. In (A), a substrate protein in the denatured conformation (red line) binds to one of the rings of wild-type GroEL. Subsequent binding of GroES to the same ring results in the release of the substrate protein into the large cavity formed by GroEL/ES (the *cis*-ternary complex), where the substrate protein can fold to its native conformation (represented by an orange oval). Binding of ATP to the opposite ring (*trans*-ring) triggers the release of GroES and substrate protein. In (B), the single-ring variant with an ATPase deficient mutation, SR398, binds GroES in the same manner as wild-type GroEL. The absence of the *trans*-ring results in formation of a stable ternary complex. A 12-residue GroEL binding-tag (SBP sequence, shown in

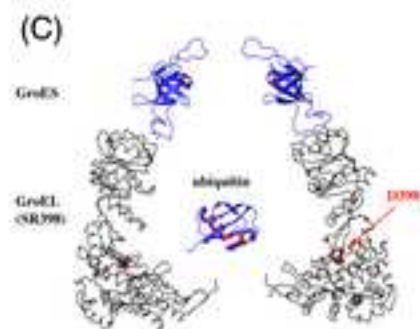
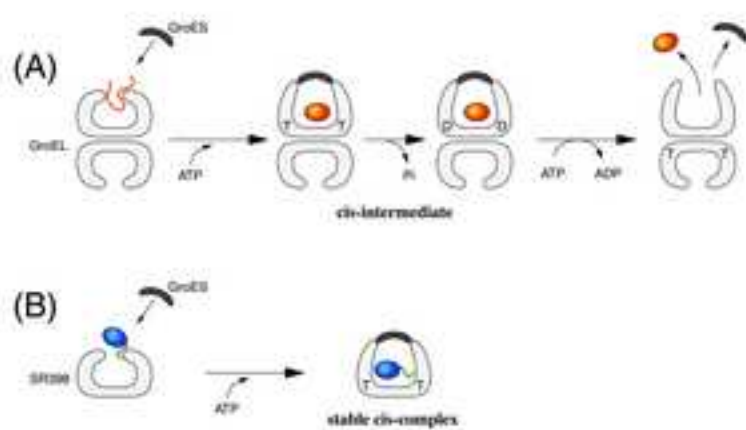
green) enables binding of substrate protein in its native conformation (represented by a blue oval). ‘T’ and ‘D’ in the cartoon designate the ATP- and ADP-bound states of GroEL, respectively. In (C), the molecules are drawn to scale from crystal structure coordinates (PDB ID 1AON and 1UBQ [14,23]) showing the volume of the chamber ( $175,000 \text{ \AA}^3$ , which corresponds to a globular protein of 142 kDa) is much larger than ubiquitin [24,25]. Asp398 C $\gamma$  of the ATP-binding site of GroEL is marked by a red sphere. Its location deep inside the complex shows that small compounds of similar size to ATP can freely enter the cavity. The figure was drawn using Molmol [26].

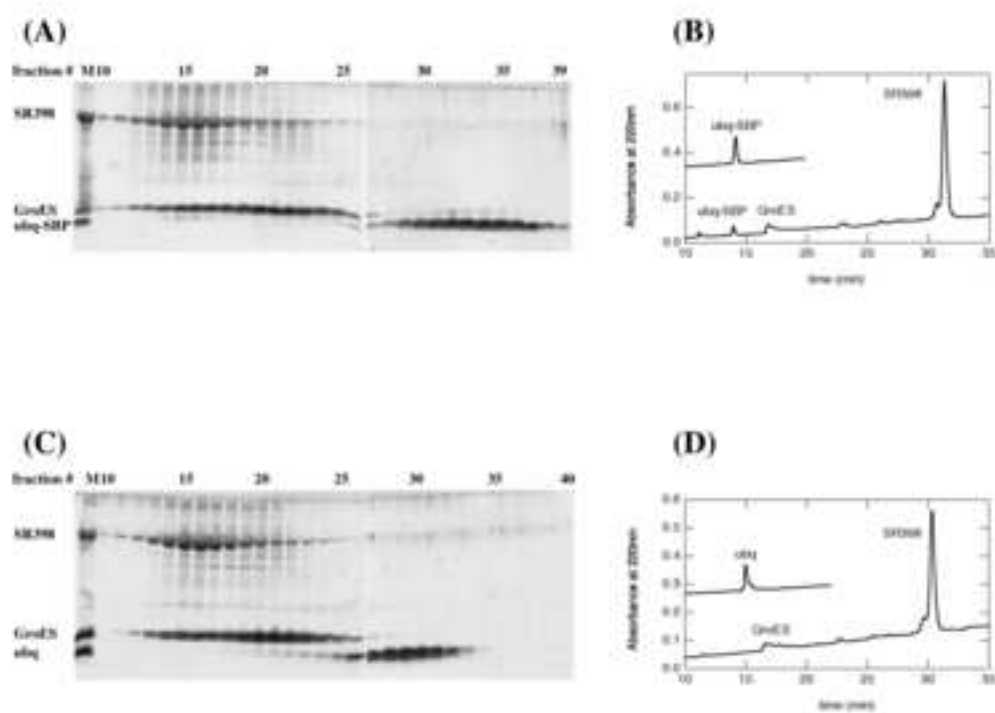
**Figure 2.** Formation of the ternary complex of SR398, GroES and substrate. (A, C) SR398, GroES, and ubq-SBP (A) or wild-type ubiquitin (C) were incubated in the presence of ATP in a molar ratio of 1:2:10. The mixture was subjected to size-exclusion chromatography. Fractions (200  $\mu\text{L}$  each) were collected and analyzed by SDS-PAGE. (B, D) Fractions 14-17 of the size-exclusion chromatography run shown in (A) or (C) were concentrated and applied to reversed-phase HPLC to determine the molar ratio of SR398 and ubq-SBP (B), or to confirm the failure of the complex formation between SR398 and wild-type ubiquitin (D). The inset shows the elution profile of each substrate protein alone.

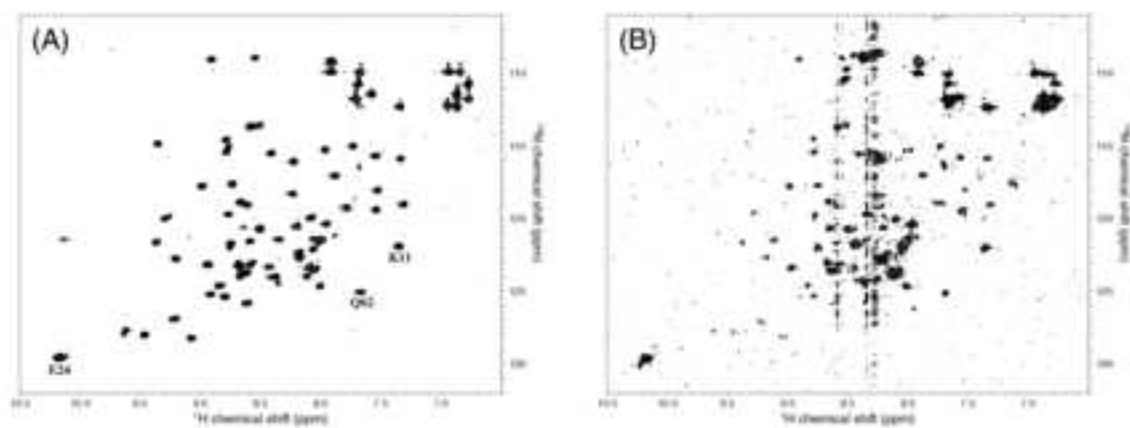
**Figure 3.**  $^{15}\text{N}$ -HSQC spectra of ubiquitin-SBP (A) free in solution and (B) encapsulated in SR398/GroES. The line widths of the peaks labeled in panel (A) are analyzed in detail in Figure 4.  $t_1$ -noise bands at the  $^1\text{H}$  chemical shifts of 8.6 and 8.3 ppm are from ATP NHs.

**Figure 4.** Cross-sections taken along the  $^1\text{H}$  frequency axis of the  $^{15}\text{N}$ -HSQC spectra shown in Figure 4. The left and right panels are taken from the spectra of the reference (Fig. 3A) and the ternary complex (Fig. 3B), respectively. The red lines show the best fits to a Lorentzian line shape. The assignments and line widths  $\Delta\nu_{\text{FWHH}}$  are indicated.

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